Biochimica et Biophysica Acta 860 (1986) 118–124
Elsevier

BBA 73214

Effect of neuraminidase treatment on the topological distribution of phospholipids in chick brain microsomes

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(Received July 1st, 1985)
(Revised manuscript received April 21st, 1986)

Key words: Phospholipid asymmetry; Sialoglycoconjugate; Neuraminidase; Microsome; (Chick brain)

The desialylation of chick brain microsomal membranes affects the transbilayer distribution of phospholipids. When intact microsomes were treated with neuraminidase, less phosphatidylcholine and sphingomyelin could be hydrolysed with phospholipase C under experimental conditions which allowed the hydrolysis of the phospholipids of the external leaflet only. In contrast, the accessibility of phosphatidylethanolamine and phosphatidylserine to the external probes (trinitrobenzene sulfonic acid or phospholipase C) was not affected. After neuraminidase treatment of a microsomal fraction, less phosphatidylcholine, newly synthesized through the cytidine pathway, could be hydrolysed by phospholipase C, whereas the reaction of newly synthesized phosphatidylethanolamine molecules with trinitrobenzene sulfonic acid was not affected. The results suggest that in biological membranes some choline phospholipid molecules may interact with the sialyl residue of sialocompounds. This interaction may contribute to the maintenance of phospholipid asymmetry in brain membranes.

Introduction

Phospholipids are the major lipid components of eukaryote membranes. In the brain they account for about 50–70% of total lipids [1,2] and it becomes more and more evident that they are distributed asymmetrically in different membrane structures, such as microsomes or plasma membranes [3–5]. The different functions of the nerve membranes require specific lipids, hence the intramembrane distribution of the different phospholipids may be of crucial importance in the control of the integration of any vectorial process occurring in these membranes. It is therefore relevant and important to know how asymmetry arises in the membranes. The mechanism by which the asymmetric distribution of the lipids is produced and maintained still remains obscure, although recent observations suggest an important role for proteins in the stabilization of the phospholipid distribution [6,7]. Freysz et al. [8,9] have reported that the enzymes involved in the de novo synthesis of phospholipids and in the interconversion reactions are differently embedded in the microsomal bilayer. However, more recent studies by Higgins [10], Coleman and Bell [11] and Freysz et al. [12] have indicated that the site of synthesis of PE and PC is located on the cytoplasmic side of the endoplasmic reticulum, suggesting that these enzymes may not be involved in the maintenance of the phospholipid asymmetry. Binaglia et al. [13]

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TNBS, trinitrobenzene sulfonic acid.

have observed that the removal of divalent cations from microsomal vesicles may produce a rearrangement of PE molecules, suggesting a role for divalent cations in maintaining the lipid asymmetry. The present study indicates the possible role of sialoglycoconjugates in the maintenance of the phospholipid asymmetry in chick brain microsomal membranes.

Material and Methods

Materials

Neuraminidase (*Arthrobacter ureafaciens,* spec. act.: 48 U/mg) was purchased from Nakarai Chemicals (Kyoto, Japan). Bovine serum albumin type V and phospholipase C of *Clostridum welchii* (spec. act.: 10 U/mg protein) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cytidine 5′-diphospho[*methyl*-^{14}C]choline (spec. act.: 60 mCi/mmol) and cytidine 5′-diphospho[^{14}C]ethanolamine (spec. act.: 28 mCi/mmol) were obtained from Amersham (U.K.).

Methods

Preparation of microsomes. Microsomes were prepared from fresh adult chick brain as reported by Dominski et al. [3] and used without storage.

Treatment of microsomes with neuraminidase. Microsomes were homogenized at a concentration of 2.5 mg protein/ml in 20 mM sodium acetate buffer (pH 5.0)/0.87% (w/v) NaCl. 1 mg microsomal protein was incubated at 37°C with 30 mU neuraminidase in a final volume of 0.5 ml for 15 min. The reaction was stopped by the addition of 0.1 ml Tris-HCl (500 mM, pH 8.0) and cooling in ice. The homogenate was centrifuged for 1 h at 105 000 × *g* to eliminate neuraminidase, and the microsomal pellet was homogenized in the appropriate buffer. Controls were treated similarly either with the omission of neuraminidase or in the presence of neuraminidase with the reaction stopped at time close to zero. Free sialic acid, and bound sialic acid of glycoproteins and gangliosides were determined by techniques reported by Harth et al. [14].

Labelling of microsomal phosphatidylethanolamine and phosphatidylcholine. Control and neuraminidase-treated microsomes were incubated with either CDP[^{14}C]ethanolamine or CDP[^{14}C]choline as reported by Freysz et al. [15,16]. The reaction was stopped by the addition of Ca^{2+} (final concentration 2.5 mM).

Determination of the distribution of phospholipids in the two leaflets of microsomal membranes. The distribution of the phospholipids in both leaflets of control and neuraminidase-treated microsomes was determined using phospholipase C and TNBS as described by Dominski et al. [3,15]. It has been shown that under the conditions used the probes do not penetrate into the vesicles and that only the phospholipids present in the external leaflet of the microsomal vesicles reacted with the probes.

Electron microscopy. Control and neuraminidase-treated microsomes were pelleted at 105 000 × *g* for 60 min and fixed in 10% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4). After post-fixation with 0.1% osmic acid, the fractions were dehydrated and embedded in araldite. Sections were cut using a Reichert Ultramicrotome and stained with uranyl acetate and lead citrate.

Results

Electron microscopic observations

The electron microscopic observation of the microsomal fraction obtained by differential centrifugation showed that the membranes are vesiculated and that ribosomes were localized on the outer surface of the vesicles, suggesting that, in the microsomal vesicles, the outer leaflet corresponds to the cytoplasmic side in situ (Fig. 1a). The neuraminidase treatment maintains microsomes in a vesiculated status. Less ribosomes were observed after treatment, suggesting that the ribosomes may be removed from the endoplasmic reticulum by neuraminidase (Fig. 1b). Moreover, the neuraminidase-treated vesicles appeared smaller than the controls. These changes did not seem to be due to a lack of integrity of the vesicle membranes, considering that [^{3}H]sucrose efflux was comparatively similar in both control and neuraminidase-treated vesicles (results not shown). The observations indicate that the microsomes thus treated remain sealed and can therefore be used for the investigation of lipid composition in the two membrane leaflets.

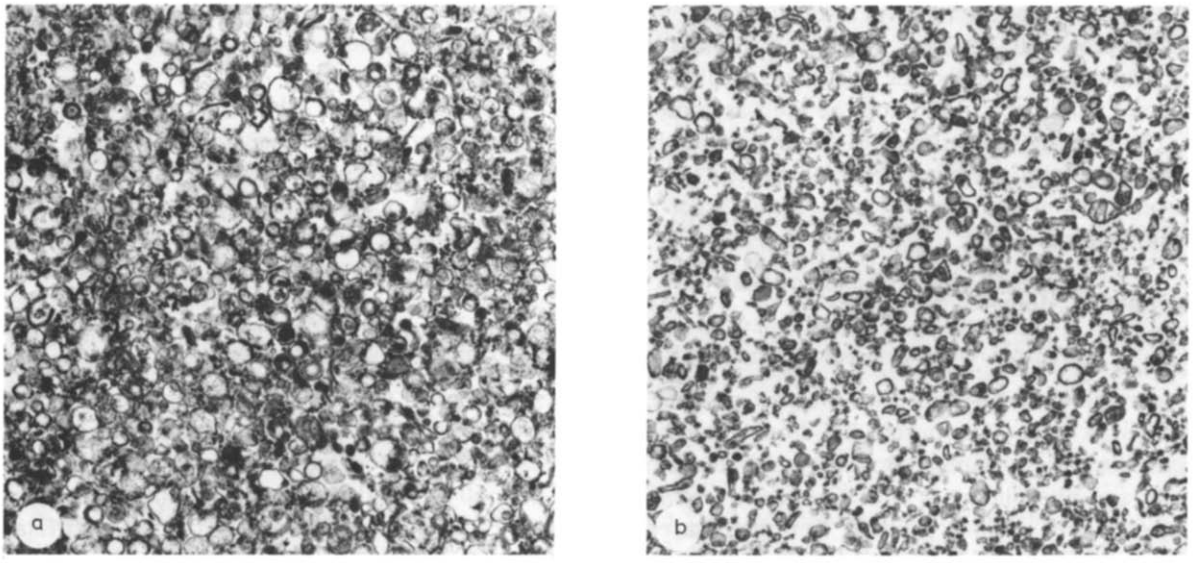

Fig. 1. Electron microscopy of microsomal vesicles: (a) control, and (b) after treatment with neuraminidase for 15 min as described in Material and Methods. ×22000.

Effect of neuraminidase on the transverse distribution of phospholipids in microsomal vesicles

The treatment of microsomes with neuraminidase produced a release of sialic acid. About 22% of the total glycoconjugate sialic acid was removed after 15 min of treatment. Approx. 56% of the free

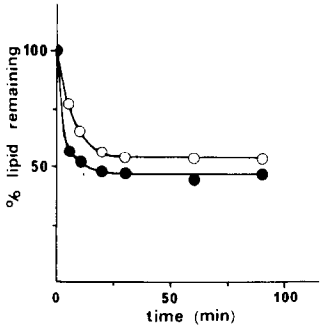

Fig. 2 (left). Hydrolysis of phospholipids of chick brain microsomes with phospholipase C of *C. welchii* as function of time. Incubations were performed as reported in Material and Methods. Percentage of total phospholipids remaining in control microsomes (●) and neuraminidase-treated microsomes (○). Each point is the average of three experiments. Standard deviation was less than 5%.

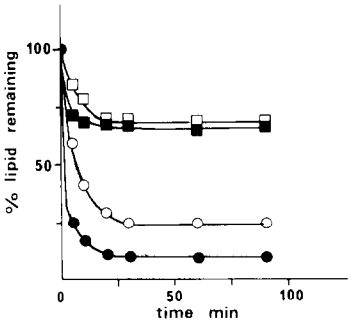

Fig. 3 (center). Hydrolysis of phosphatidylethanolamine and phosphatidylcholine of chick brain microsomes with *C. welchii* phospholipase C as a function of time. Incubations were performed as reported in Material and Methods. Closed symbols are control microsomes and open symbols neuraminidase-treated microsomes ■ and □, PE; ● and ○, PC. Results represent the percentage of phospholipids remaining. Each point is the average of three experiments. Standard deviation was less than 5%.

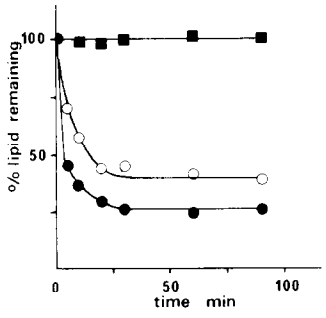

Fig. 4 (right). Hydrolysis of phosphatidylserine and sphingomyelin of chick brain microsomes with *C. welchii* phospholipase C as a function of time. Incubations were performed as reported in Material and Methods. Closed symbols are control microsomes and open symbols neuraminidase-treated microsomes. ■, phosphatidylserine; ● and ○, sphingomyelin. Results represent the percentage phospholipids remaining. Each point is the average of three experiments. Standard deviation was less than 5%.

sialic acid produced originated from glycoproteins and 44% from polysialogangliosides, a result which is in agreement with previous observations [8]. Prolonged incubation of microsomes with neuraminidase produced a release of more sialic acid, but the effect on the phospholipid distribution has not yet been investigated.

The treatment of control microsomes with phospholipase C led to the hydrolysis of about 53% of the phospholipids in 30 min (Fig. 2). No further degradation could be observed up to 90 min. When neuraminidase-treated microsomes were incubated with phospholipase C the hydrolysis of the phospholipids also reached a plateau after 30 min, but only about 46% were degraded, indicating that less phospholipids were accessible to phospholipase C.

The determination of the hydrolysis of the different microsomal phospholipids by phospholipase C is reported in Figs. 3 and 4. The results show that in both control and neuraminidase-treated microsomes the hydrolysis of PC, PE and sphingomyelin reached a plateau after 30 min. PS and phosphatidylinositol were not degraded at all, in agreement with previous observations [3], indicating that the phospholipase C used did not act on these two phospholipids. The comparison of the hydrolysis of the different phospholipids on control and neuraminidase-treated microsomes showed that PC and sphingomyelin were less accessible to the enzyme in desialylated microsomes. In neuraminidase-treated microsomes only about 75% of PC and 56% of sphingomyelin could be hydrolysed, versus 90% and 71% in control vesicles. However, no differences in the accessibility of PE to the phospholipase C were observed, the amount hydrolysed being 34 and 33% for control and neuraminidase-treated microsomes, respectively.

Moreover, when microsomes were exposed to TNBS in non-penetrating conditions about 32% of the PE and 10% of the PS reacted with the probe (results not shown). These results indicated that the decreased accessibility of choline phospholipids to the phospholipase C may not be due to the interference of the enzyme with neuraminidase because: (a) the neuraminidase was removed from the incubation mixture prior treatment with phospholipase C; and (b) the hydrolysis of phosphatidylethanolamine with phospholipase C was not affected by the treatment of microsomes with neuraminidase, and the amount of phosphatidylethanolamine hydrolysed was similar to the amount which reacted with TNBS in non-penetrating conditions. Thus, the results summarized in Table I suggest that the partial removal of sialic acid from the external side of the microsomes produced a translocation of choline phos-

TABLE I

EFFECT OF NEURAMINIDASE ON THE TRANSVERSE DISTRIBUTION OF PHOSPHOLIPIDS IN CHICK BRAIN MICROSOMAL MEMBRANES

Microsomes (1 mg protein) were incubated with or without neuraminidase for 15 min. The determination of the phospholipids in the outer and inner leaflet was performed as described by Dominski et al. [3] using phospholipase C of *C. welchii* and TNBS. Each value represents the average of three different experiments. Standard variation was less than 10%.

	Control microsomes (23.4 nmol bound sialic acid/mg protein)			Neuraminidase-treated microsomes (18.4 nmol bound sialic acid/mg protein)		
	whole microsomes (nmol/mg protein)	outer leaflet (%)	inner leaflet (%)	whole microsomes (nmol/mg protein)	outer leaflet (%)	inner leaflet (%)
Total phospholipids	803.5	–	–	803.5	–	–
Phosphatidylcholine	340.3	89.4	10.6	318.2	74.6	25.4
Sphingomyelin	74.3	71.4	28.6	71.3	56.3	43.7
Phosphatidyl-ethanolamine	260.3	34.0	66.0	276.2	33.0	67.0
Phosphatidylserine	77.0	10.0	90.0	80.4	10.0	90.0

TABLE II

EFFECT OF NEURAMINIDASE ON THE DISTRIBUTION OF NEWLY SYNTHESIZED PHOSPHATIDYLCHOLINE IN CHICK BRAIN MICROSOMAL MEMBRANES

Control and neuraminidase-treated microsomes were incubated with CDP[^{14}C]choline as reported by Freysz et al. [15,16]. The reactions were stopped by addition of Ca^{2+} and the distribution of labelled phosphatidylcholine in the inner and outer leaflet was determined using phospholipase C. Results are expressed in pmol PC synthesized per mg protein. Each value represents duplicate determinations of two different experiments. Standard variation was less than 10%.

Time of incubation (min)	Control microsomes			Neuraminidase-treated microsomes		
	A: outer leaflet	B: inner leaflet	ratio A/B	A: outer leaflet	B: inner leaflet	ratio A/B
15	1489.7	188.5	7.90	1999.6	655.4	3.05
60	3285.7	414.0	7.94	4999.0	1321.0	3.78

pholipids from the external to the internal leaflet, but no translocation of aminophospholipids.

Effect of neuraminidase on the distribution of newly synthesized phospholipids in chick brain microsomes

In order to obtain some information on the effect of sialoglycoconjugates on the transmembrane movement of phospholipids, control and neuraminidase-treated microsomes were incubated with CDP[^{14}C]choline or CDP[^{14}C]ethanolamine and the intramembrane localization of newly synthesized PC and PE was investigated. Table II shows that the synthesis of PC was slightly enhanced in neuraminidase-treated microsomes and that much less of the newly synthesized molecules was accessible to phospholipase C. In control microsomes about 11% of the newly synthesized PC molecules were not hydrolyzed by phospholipase C, whereas in neuraminidase-treated microsomes about 21–25% were not hydrolysed.

On the other hand, when microsomes were incubated with CDP[^{14}C]ethanolamine the synthesis of phosphatidylethanolamine and the corresponding plasmalogens was slightly reduced in neuraminidase-treated microsomes. However, the percentage of newly synthesized ethanolamine phospholipids reacting with TNBS (33% for phosphatidylethanolamine and 31% for ethanolamine plasmalogens) was similar in both types of microsomes (Table III). These results suggest that the removal of sialic acid moieties from microsomal glycoconjugates produced a facilitation of the

TABLE III

EFFECT OF NEURAMINIDASE ON THE DISTRIBUTION OF NEWLY SYNTHESIZED ETHANOLAMINE PHOSPHOLIPIDS IN CHICK BRAIN MICROSOMAL MEMBRANES

Control and neuraminidase-treated microsomes were incubated with CDP[^{14}C]ethanolamine as reported by Freysz et al. [15,16]. The reactions were stopped by addition of Ca^{2+} and the distribution of labelled ethanolamine phospholipids in the inner and outer leaflet was determined using TNBS. Results are expressed in pmol PE synthesized per mg protein. Each value represents duplicate determinations of two different experiments. Standard variation was less than 10%.

Time of incubation (min)	Control microsomes			Neuraminidase-treated microsomes		
	A: outer leaflet	B: inner leaflet	ratio A/B	A: outer leaflet	B: inner leaflet	ratio A/B
Phosphatidylethanolamine						
15	552.0	269.9	2.04	496.1	236.7	2.10
60	749.4	389.6	1.92	589.2	290.2	2.09
Ethanolamine plasmalogens						
15	252.1	108.7	2.32	230.0	99.6	2.31
60	373.1	168.5	2.21	276.3	127.8	2.16

transfer of newly synthesized molecules of PC but not of PE from the outer to the inner leaflet of the microsomal vesicles.

Discussion

The main question regarding the phospholipid asymmetry in biological membranes concerns the mechanisms involved in the generation and maintenance of this asymmetry. Since the asymmetry is not an inherent characteristic of phospholipid bilayers except in liposomes [17,18], it should be assumed that the interaction of the lipids with other membrane components may play a determining role in the maintenance of the transverse distribution of phospholipids. Many proteins require specific phospholipids for their maximal biological activity, and therefore the intrinsic localization of proteins in membranes and their interaction with lipids may be of great importance. Numerous studies [4,19–22] on lipid-protein interaction have shown that the lateral diffusion and the transverse translocation of phospholipids are affected by proteins. However the mechanisms which control these movements still remain unclear. The phospholipids may interact with proteins either through their polar head group and/or through the apolar moiety of their acyl chains [23]. The present results show that, in microsomal vesicles treated with neuraminidase, less PC and sphingomyelin were hydrolysed by phospholipase C, whereas the accessibility of PE and PS to this enzyme or to TNBS was not affected. The data suggest that the removal of sialic acid residues from the microsomal membrane produces a translocation of PC and sphingomyelin molecules from the outer to the inner leaflet, but has no effect on the transbilayer movements of PE and PS molecules.

Since sialic acid and choline have opposite charges, the sialoglycoconjugates and choline phospholipids may form electrostatic interactions which may participate in the asymmetric maintenance of the phospholipids in membranes. However, the ratio of sialic acid residues to choline residues is about 1–35 in microsomal membranes, indicating that all the choline phospholipid molecules cannot interact with the sialyl residues. Therefore other mechanisms like hydrophobic interactions may be involved in the maintenance of the phospholipid asymmetry. Such interactions have been studied in liposomes and biological membranes and various effects on their physicochemical properties have been observed [24–26]. The treatment of microsomes with neuraminidase produced asialo-compounds which may disturb the organization of the bilayer. Thompson et al. [27] reported that gangliosides are distributed randomly in membrane bilayers, whereas asialoglycolipids are largely concentrated in a phase-separated domain system like clusters. Such a phase transition between gangliosides and asialoglycolipids may produce a rearrangement of the phospholipid bilayer and especially of those phospholipids located in the neighbourhood of the sialoglycoconjugates. This hypothesis would imply that the sialoglycoconjugates are preferentially surrounded with choline phospholipids. Nevertheless, whatever the nature of the interactions between sialoglycoconjugates and phospholipids in biological membranes may be, it seems likely that the gangliosides and/or the sialoglycoproteins may contribute in part to stabilize the position of choline phospholipids in the outer leaflet of microsomal vesicles. The question as to whether choline phospholipids interact preferentially with sialoglycolipids or sialoglycoproteins remains unsolved. In the hypothesis of an electrostatic interaction between the sialic acid residues of sialoglycoconjugates and choline phospholipids, it seems likely that PC and sphingomyelin would preferentially interact with the sialyl residues of gangliosides, which have in general a position much closer to the polar head group of phospholipids than those of sialoglycoproteins in biological membranes.

The microsomal fractions prepared from chick brain showed the properties of fractions enriched in endoplasmic reticulum [3]. The assay of some marker enzymes showed a little contamination with plasma membranes [3], which are known to contain high amounts of sialoglyconjugates, mostly located in the external leaflet [28]. Therefore the effect of the removal of sialyl residues from membrane components on the transbilayer movement of choline phospholipids may be restricted essentially to the plasma membranes. However, recent investigations revealed the presence of sialoglyco-

conjugates in intracellular membranes [28], and thus the results obtained with a microsomal fraction may represent an indication of the overall transbilayer movement in these different membrane vesicles.

Acknowledgements

We are grateful to C. Marchand and M. Miehe for their excellent technical assistance and to C. Thomassin-Orphanides for manuscript preparation. This work was supported by grants from the Italian CNR (grant CT 810012504) and from NATO (grant 07582).

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